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## Abstract

This study has elucidated the fragmentation pathway for deprotonated isoflavones in electrospray ionization using MSn ion trap mass spectrometry and triple quadrupole mass spectrometry. Genistein-d4 and daidzein-d3 were used as references for the clarification of fragment structures. To confirm the relationship between precursor and product ions, some fragments were traced from MS2 to MS5. The previous literature for the structurally related flavones and flavanones located the loss of ketene ($C_2H_2O$) to ring C, whereas the present fragmentation study for isoflavones has shown that the loss of ketene occurs elsewhere at ring A. In the further fragmentation of the $[M-H-CH_3]^{\bullet-}$ radical anion of methoxylated isoflavones, loss of a hydrogen atom was commonly found. [M-H-CH3□CO□B-ring]- could be a characteristic fragment ion of glycitein and be used to differentiate glycitein from its other isomers. Neutral losses of CO and $CO_2$ were prominent in the fragmentation of deprotonated anions in the ion trap mass spectrometry, whereas recyclization cleavage accounted for a very small proportion. In comparison with triple quadrupole mass spectrometry the use of the ion trap MSn mass spectrometry has the advantage of better elucidation of the relationship between precursor and product ions.



## Keywords

CMMB


## Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences


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# A fragmentation study of isoflavones in negative electrospray ionization by $MS^n$ ion trap mass spectrometry and triple quadrupole mass spectrometry

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This study has elucidated the fragmentation pathway for deprotonated isoflavones in electrospray ionization using $MS^n$ ion trap mass spectrometry and triple quadrupole mass spectrometry. Genistein-$d_4$ and daidzein-$d_3$ were used as references for the clarification of fragment structures. To confirm the relationship between precursor and product ions, some fragments were traced from $MS^2$ to $MS^5$. The previous literature for the structurally related flavones and flavanones located the loss of ketene ($C_2H_2O$) to ring C, whereas the present fragmentation study for isoflavones has shown that the loss of ketene occurs elsewhere at ring A. In the further fragmentation of the $[M–H–CH_3]^-$ radical anion of methoxylated isoflavones, loss of a hydrogen atom was commonly found. $[M–H–CH_3–CO–B\text{-ring}]^-$ could be a characteristic fragment ion of glycitein and be used to differentiate glycitein from its other isomers. Neutral losses of CO and $CO_2$ were prominent in the fragmentation of deprotonated anions in the ion trap mass spectrometry, whereas recyclization cleavage accounted for a very small proportion. In comparison with triple quadrupole mass spectrometry the use of the ion trap $MS^n$ mass spectrometry has the advantage of better elucidation of the relationship between precursor and product ions.

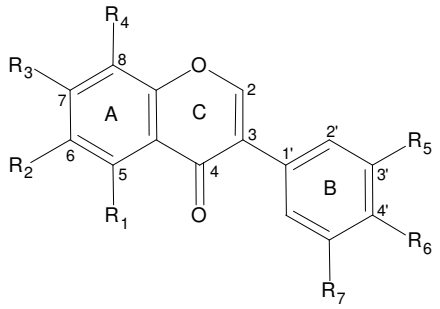
## INTRODUCTION

Isoflavones are the most well known class of phytoestrogens with functional estrogenic and antiestrogenic action and structural similarity to mammalian estrogenic hormones. These compounds are primarily found in the *Fabacease* family, and are distributed in edible plants and derived products.[1, 2] Isoflavones belong to a subclass of the flavonoids.[3] In the past decade many analytical methods for the identification and quantitation of flavonoids in plant derived products and biological matrices have been reported.[3-5] Among them mass spectrometry coupled with high performance liquid chromatography (HPLC) has proved to be one of the most effective techniques particularly for the analysis of complex mixtures in biological samples.[5, 6] For this reason, a number of papers dealing with the fragmentation mechanism of a range of flavonoids, mainly flavones, flavanones and flavonols, have been published.[7-21] Ma et al[7, 8] analysed the fragmentation behavior of flavones, flavonols and methoxyflavones in positive ion mode using fast-atom bombardment and collision-induced dissociation tandem mass spectrometry. Fabre et al[9] proposed a fragmentation scheme and product ion structures for flavone, flavonol, and flavanone aglycones in negative ion mode. Kuhn et al[10] found a characteristic double neutral loss of CO at ring C for flavonoid type compounds. March et al[11, 12] studied fragmentation scheme of a flavonol and an

isoflavone glycoside using electrospray quadrupole time-of flight mass spectrometry in both positive and negative mode, and successfully explained a fragmentation mechanism by an intermediate structure of seven-membered ring C. Justesen[13] examined the fragmentation rule of methoxylated flavones and flavonols in negative ion mode, and found that the loss of a methyl group (−15u) was the characteristic fragmentation.

The structures of isoflavones differ from the isomeric flavones by the position of the ring B, which could lead to significantly different fragmentation behavior to the flavones, flavanones and flavonols. In a previous paper we have described a robust method for the simultaneous identification and quantitation of isoflavones and lignans.[22] Given the importance of isoflavones to a number of research areas and the inreasing use of MS techniques for their identification and quantitation a clear understanding of the fragmentation behavior of isoflavones is needed. To our knowledge, no systematic study has been reported before for the fragmentation scheme and structures on isoflavones in negative ESI mode by step $MS^n$ fragmentation using ion trap mass spectrometry.

The aim of the present study was therefore to elucidate the fragmentation pathway of isoflavones (genistein, daidzein, biochanin A, formononitin, and glycitein; see Figure 1) in negative ESI mode by $MS^n$ ion trap mass spectrometry, with the aid of two deuterated compounds. This enhanced understanding will, in addition, aid further structural identification of other flavonoids in mass spectrometric analysis.




| No. | Isoflavone | $[M-H]^-$ | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | $R_6$ | $R_7$ |
|---|---|---|---|---|---|---|---|---|---|
| **1** | Genistein | 269 | OH | H | OH | H | H | OH | H |
| **2** | Genistein-$d_4$ | 273 | OH | D | OH | D | D | OH | D |
| **3** | Daidzein | 253 | H | H | OH | H | H | OH | H |
| **4** | Daidzein-$d_3$ | 256 | H | H | OH | D | D | OH | D |
| **5** | Biochanin A | 283 | OH | H | OH | H | H | $OCH_3$ | H |
| **6** | Formononetin | 267 | H | H | OH | H | H | $OCH_3$ | H |
| **7** | Glycitein | 283 | H | $OCH_3$ | OH | H | H | OH | H |

**Figure 1**. Structures of the isoflavones studied in this paper.

## EXPERIMENTAL

### Materials

Standards of isoflavones: daidzein (98% purity), genistein (98% purity), formononetin (99% purity), biochanin A (97% purity), and glycitein (97% purity) were purchased from Sigma–Aldrich (Sydney, Australia). Deuterated genistein(3',5',6,8-

$d_4$) (98% purity, 95% isotopic enrichment) and deuterated daidzein (3',5',8-$d_3$) (98% purity, 97% isotopic enrichment) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Chemical structures and trivial names of all the standard isoflavones are shown in Figure 1. Acetonitrile and methanol, both HPLC grade, were supplied by Crown Scientific (Sydney, Australia). Milli-Q water (Milli-Q plus 185, Australia) was used for making up all aqueous solutions. Standard stock solutions of each compound were prepared at a concentration of 100µg/mL in acetonitrile or acetonitrile plus 20% methanol. Working solutions were prepared in acetonitrile/water (1:3, v/v) and obtained by tenfold dilution to a concentration of 10µg/mL. The solutions was infused to the ESI source by the syringe pump of the mass spectrometer, using a 500-µl Unimetrics syringe at a flow rate of 10µl $min^{-1}$ (for $MS^2$ and $MS^3$ experiment) and 60µl $min^{-1}$ (for $MS^4$ and $MS^5$ experiment).

**Mass spectrometry**

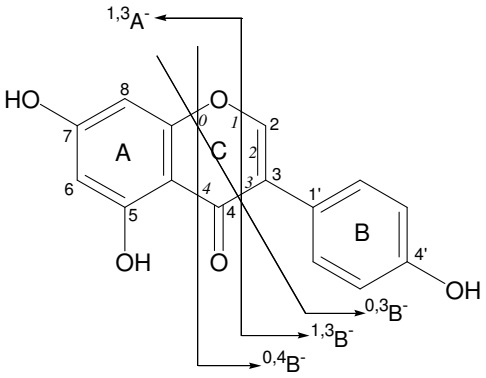
The fragmentation experiments by ion trap mass spectrometry were performed using a ThermoElectron Finnigan LTQ linear ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an electrospray ionization source. Standard solutions were directly infused into the LTQ linear ion trap mass spectrometer, via an ESI source. All the mass spectra were acquired in negative ion mode with the spray voltage 3.61 kV, capillary voltage at -11.83V, capillary temperature 274.8°C. Nitrogen was used as both the sheath and auxiliary gas at 29 and 3 arbitrary units respectively. Helium was used as a damping and collision gas at a partial pressure of 0.1 Pa. The relative collision energies for each compound were from 36% to 49%, respectively. The data range utilized was from 80 to 300u.

The fragmentation experiments by triple quadrupole mass spectrometry were performed using a Micromass Quattro Micro triple quadrupole mass spectrometer (Micromass, Manchester, UK) with an electrospray ionization source. Data acquisition was in a negative mode. The electrospray source parameters were fixed as follows: electrospray capillary voltage 3.0kV, cone 40eV, source temperature 100°C, desolvation temperature 120°C, and desolvation gas $N_2$ at 300 L/hr. The collision energies for each compound were from 30 to 35 eV, respectively. Spectra were recorded in the range 100–300u.

## RESULT AND DISCUSSION

**Nomenclature**

In order to clarify the fragmentation patterns obtained, particular nomenclature needed to be introduced to define the fragment ions involving cleavage of two bonds of the ring C. In this paper the nomenclature adopted for the recyclization cleavages was adapted from the one proposed by Y. L. Ma and co-workers.[7] The $^{i,j}A$ and $^{i,j}B$ labels refer to the product ions containing intact ring A and ring B, respectively, in which the superscripts i and j indicate the ring C bonds that have been broken (see Scheme 1).




**Scheme 1**. Nomenclature adopted for defining retrocyclization cleavages observed in this study.

**Fragmentation of genistein and daidzein in ion trap mass spectrometry**

Generally the neutral losses of CO, $CO_2$, $C_3O_2$, and ketene ($C_2H_2O$) are prominent in the ion trap mass spectrometry, whereas the ring C retrocyclization cleavages accounted for only a small proportion. To observe the effect of relative collision energy on fragmentation patterns, a series of relative collision energies was applied to the fragmentation of compound **1** (genistein), $MS^2$ experiments showed that a consecutive increase of relative collision energy in the ion trap mass spectrometer did not change the abundance proportion of each product ion peak, but only reduced the abundance of the precursor ion at the beginning. The fragmentation behavior of isoflavones was found to be similar to that for flavones and flavanones described by Fabre et al[9] but with some significant differences. The product ion mass spectra of $[M–H]^–$ of compounds **1** (genistein) and **2** (genistein-$d_4$) are shown in Figure 2, and their product ions are shown in Table 1.

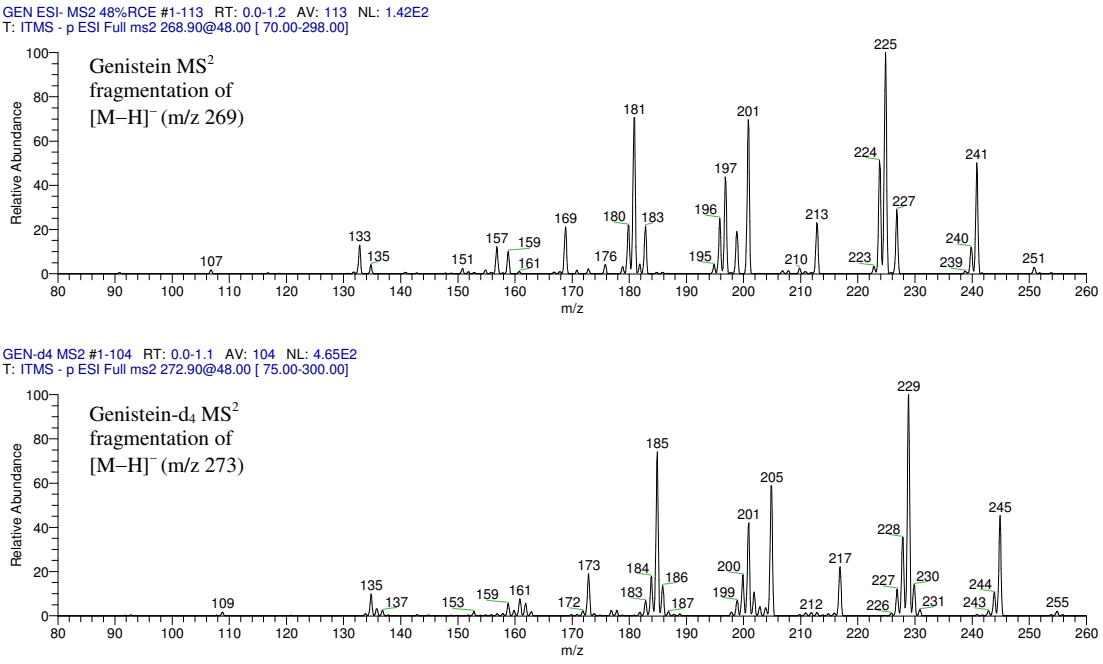



**Figure 2**. Fragmentation spectra of $[M–H]^–$ of compounds **1** and **2**.

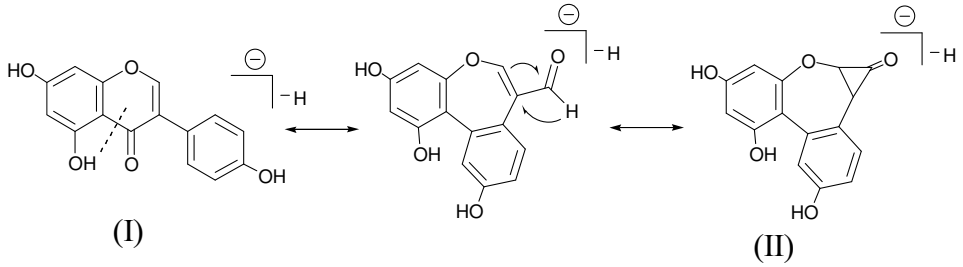
**Table 1.** Product ions obtained from $[M–H]^–$ ions in ion trap $MS^2$ spectra for compounds **1** and **2**[*m/z* with relative abundances (%) in parenthesis].

| **Ions** | **1** | **2** |
|---|---|---|
| $[M–H–CO]^–$ | 241 (50) | 245 (50) |
| $[M–H–C_2H_2O]^–$ | 227 (30) | 230 (15) |

| | | |
|---|---|---|
| $[M–H–CO_2]^-$ | 225 (100) | 229 (100) |
| $[M–H–2CO]^-$ | 213 (25) | 217 (25) |
| $[M–H–C_3O_2]^-$ | 201 (70) | 205 (60) |
| $[M–H–CO_2–CO]^-$ | 197 (45) | 201 (40) |
| $[M–H –C_2H_2O–CO_2]^-$ | 183 (20) | 186 (15) |
| $[M–H–2CO_2]^-$ | 181 (70) | 185 (70) |
| $[M–H–2CO–CO_2]^-$ | 169 (20) | 173 (20) |
| $[M–H–C_3O_2–C_2H_2O]^-$ | 159 (10) | 162 (3) |
| $^{0,3}B^-$ | 133 (15) | 135 (10) |
| $^{0,3}A^-$ | 135 (5) | 137 (2) |
| $^{1,3}A^-$ | 151 (2) | 153 (1) |
| $^{1,3}A^–CO_2$ | 107 (1) | 109 (1) |

*48% of the relative collision energy was used in the fragmentation.

March et al[12] proposed a seven-membered ring C structure, which successfully explained the mechanism of eliminating $CO_2$ at ring C on an isoflavone glycoside $[Y_0–H]^-$ radical anion. We have found this to be useful and have adapted this idea to the isoflavone $[M–H]^-$ ion, proposing a similar seven-membered ring C structure with a cycloacetone. This intermediate structure (structure II) is thought to be formed as part of the process of ring C loss of $CO_2$ (or sometimes CO or CHO·). Scheme 2 depicts this structural conversion. The formation of the seven-membered ring C was described before.[12]



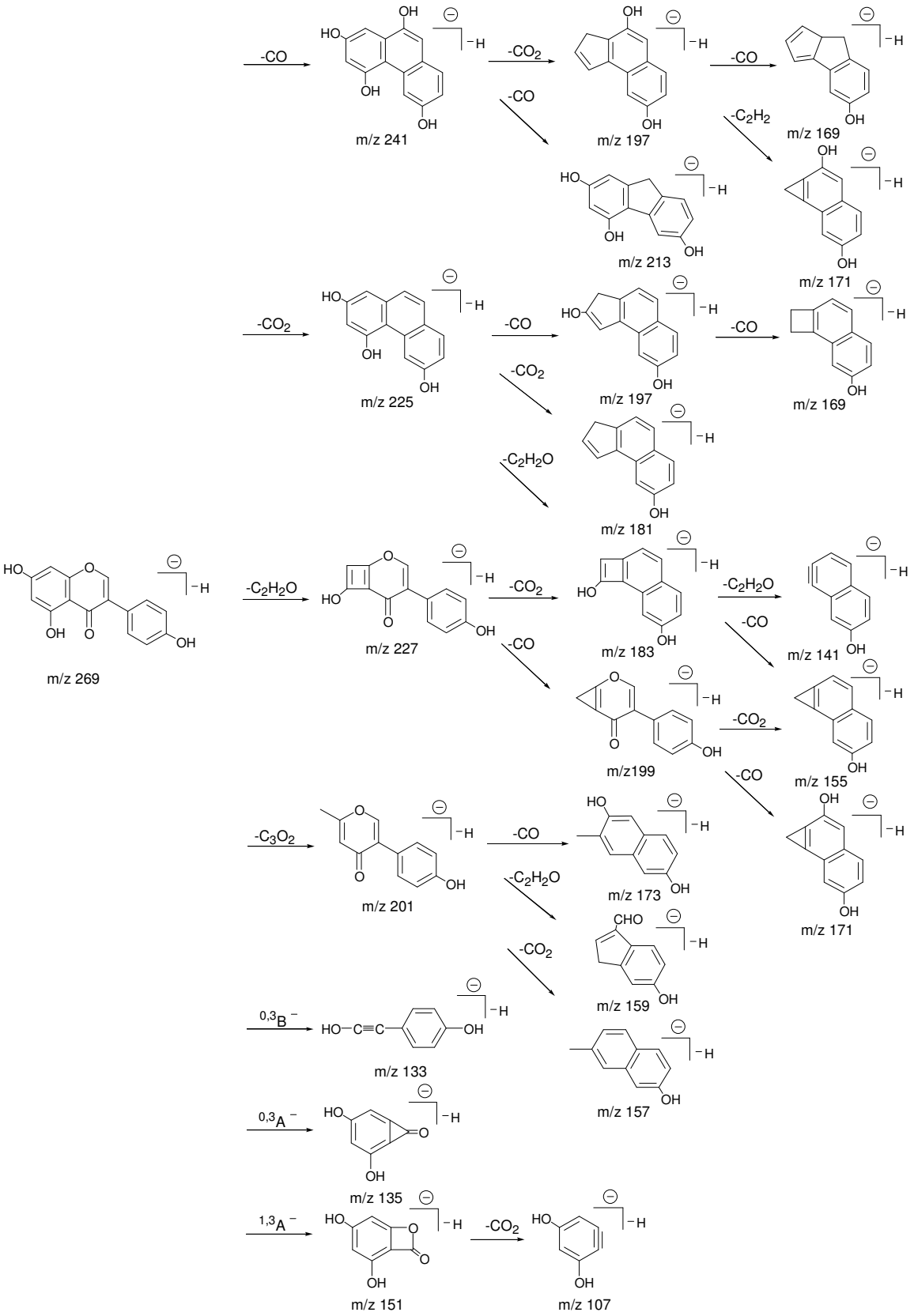

(I) (II)

**Scheme 2**. Change of structure of isoflavone before neutral loss at ring C.

The fragmentation scheme of compound **1** (genistein) is shown in Scheme 3. The fragmentation pathways in this paper are on the basis of stepwise $MS^n$ fragmentation experiments of the major product ions of each compound; the proposed structures of fragment ions are plausible on the basis of chemical intuition and are assigned tentatively.

The loss of CO and $CO_2$ at ring C by the structure II yields $[M–H–CO]^-$(m/z 241) and $[M–H–CO_2]^-$(m/z 225) respectively. Both these ions possess stable structures with a basic phenanthrene framework. Loss of $CO_2$ from m/z 241 yields $[M–H–CO–CO_2]^-$ , and whereas CO loss from m/z 225 yields $[M–H–CO_2–CO]^-$ ; both these ions are at the same m/z 197, but their $MS^4$ spectra are quite different (see Figure 3). The spectrum from $[M–H–CO–CO_2]^-$ gives an obvious fragment peak of m/z 171, formed by a loss of $C_2H_2$. These two m/z 197 ions clearly possess different structures, stemming from differences of their precursor ions. In fragmentation of flavonoids, the loss of $CO_2$ is usually attributed to the same ring,[9, 12, 18, 21] and the two oxygen atoms need to be separated by no more than two carbon atoms,[12] so the $CO_2$ loss in compound **1** could only involve either ring A or ring C. For this reason, the m/z 241 could be only formed by the loss of CO at the ring C, and further losses of $CO_2$ at ring A to form m/z 197. If this were not the case the structure of the product ion m/z 197 from m/z 241 would be same as that from m/z 225. This is not true, as shown by the different spectra obtained for the two m/z 197 ions (Figur 3). The supposition that m/z 225 is formed by loss of $CO_2$ at the ring C is confirmed by its further fragmentation behavior. $MS^3$ spectrum of m/z 225 gives $[M–H–CO_2–C_2H_2O]^-$ (m/z 183) peak, and its corresponding ion m/z 229 in compound **2** (genistein-$d_4$) gives $[M–H–CO_2–C_2HDO]^-$ (m/z 186). This means that the loss of ketene is not at the ring C because there is no deuterium atom at ring C and therefore this indicates that the previous loss of $CO_2$ which yielded the m/z

225 ion definitely occurred at the ring C. All these facts lead to the proposed structures of m/z 241 and m/z 225, in which ring A is kept intact (see Scheme 3).




**Scheme 3**. Proposed fragmentation scheme of genistein in ESI- ion trap mass spectrometry.

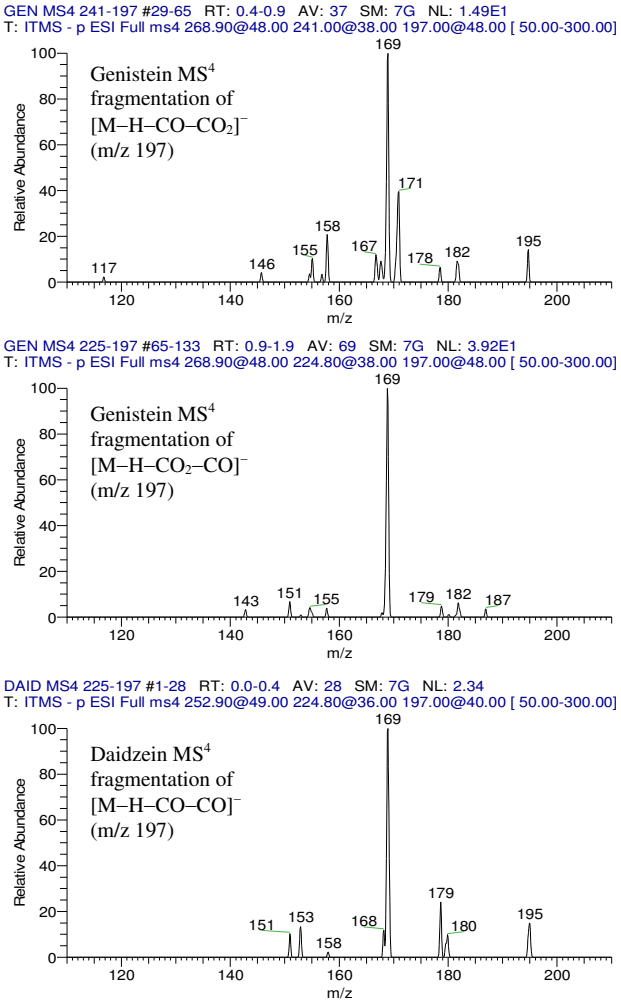



**Figure 3**. Fragmentation spectra of three m/z 197 product ions.

Fabre et al[9] found that the loss of ketene ($C_2H_2O$) in flavones and flavanones occurred at ring C. Our experiment using the corresponding deuterated compounds revealed that in isoflavones the loss of ketene occurred at the ring A, This supposition is further supported by the formation of $[M–H–C_2H_2O]^-$ ion (m/z 227) and its corresponding $[M–H–C_2HDO]^-$ ion (m/z 230). $MS^3$ fragmentation spectra of these two ions are identical except a difference of 3u (see Figure 4). Scheme 4 shows the formation of these fragments.

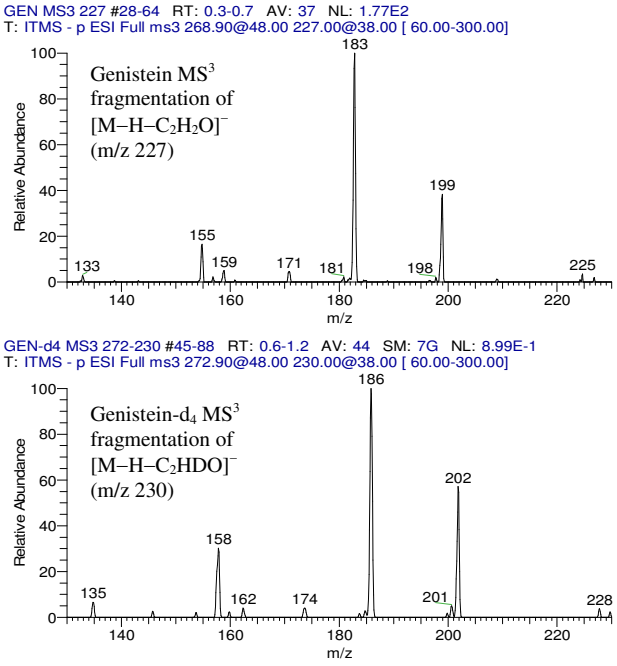
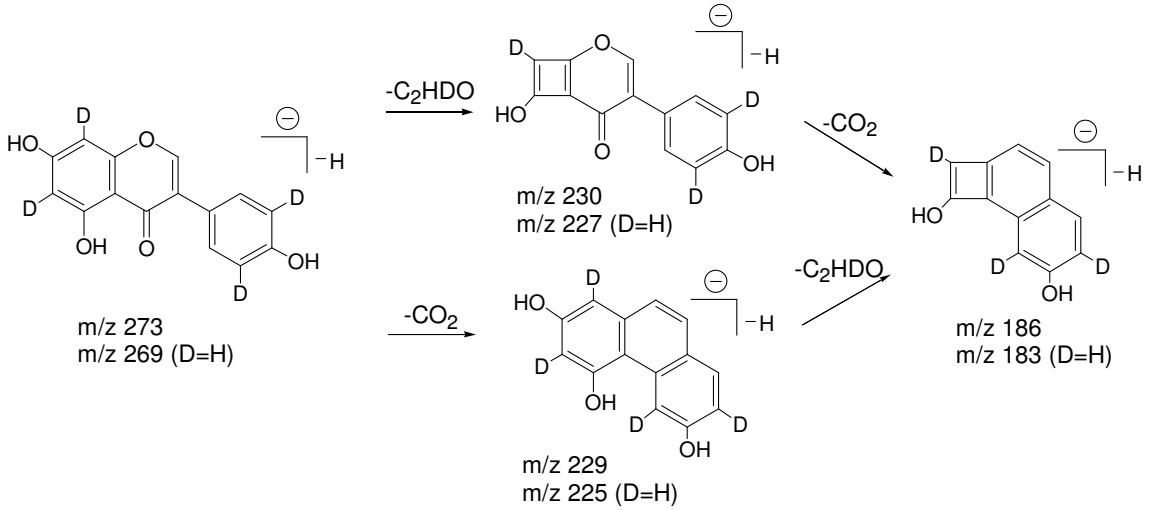



**Figure 4**. Fragmentation spectra of m/z 227 and its corresponding deuterated anion m/z 230.




**Scheme 4**. Formation of ion through loss of ketene in the deuterated fragment.

It is worth mentioning that the idea that the losses of ketene and $C_3O_2$ occurred at the ring A and not ring B is supported by the fact that m/z 133 ion corresponding to a $^{0,3}B^-$ cleavage is found in $MS^3$ fragmentation of both m/z 227 and m/z 201 ions. This indicates that after the loss of ketene and $C_3O_2$ both ions still keep the ring B intact, for which the conventional structures (structure I) with intact ring C and ring B are accepted for m/z 227 and m/z 210 ions as shown in their proposed structures in Scheme 3.

Figure 5 shows the fragmentation spectra of $[M–H]^-$ ions for compounds **3** (daidzein) and **4** (daidzein-$d_3$), and their proposed product ions are shown in Table 2. In the $MS^2$ spectrum of daidzein m/z 224 peak is distinct (about 90% of that of the base peak, see Figure 5). This radical anion could be formed by either loss of 29u (–CHO·) from m/z 253 or loss of a hydrogen atom from m/z 225. Further $MS^3$ fragmentation of m/z 225 does not give any obvious peak at m/z 224, which suggests the latter is not likely. Therefore formation by loss of CHO· from its precursor $[M–H]^-$ ion (m/z 253) is more

likely. Further losses of CO from m/z 224 produces m/z 196, with a consecutive loss of a hydrogen atom yielding m/z 195 (see Figure 6). Scheme 5 details the proposed fragmentation scheme of daidzein in the ESI- ion trap mass spectrometry. The presence of species at m/z 225 and m/z 224 is supported by their $MS^3$ fragmentation spectra. The $MS^3$ spectra of both ions do not reveal peaks for further loss of $CO_2$, which means one of the two oxygen atoms at ring C has already been lost. So it confirms there are losses of CO and CHO· from ring C which gives peaks at m/z 225 and 224 respectively. There is no fragment corresponding to a loss of $C_3O_2$ (−68u) found for compounds **3** and **4**. This is consistent with the result for flavones where the loss of $C_3O_2$ implies a β-dihydroxyl configuration in the ring A.[9] Similarly to compound **1** (genistein), the loss of ketene does not occur at ring C but at ring A. This is supported by the formation of $[M–H–C_2H_2O]^-$ (m/z 211) and its corresponding ion in daidzein-$d_3$ $[M–H–C_2HDO]^-$ (m/z 213), and further confirmed by the fact that fragment peaks of $CO_2$ loss and $^{0,3}B^-$ cleavage are found in the further $MS^3$ spectrum of m/z 211 ion. In addition, loss of CO from m/z 225 produces m/z 197 and the $MS^4$ spectrum of this $[M–H–CO–CO]^-$ ion is different from the m/z 197 ions observed in genistein (see Fig 3). It may be proposed that this ion is another isomer of m/z 197 with intact ring A and ring B in its structure (see Scheme 5).

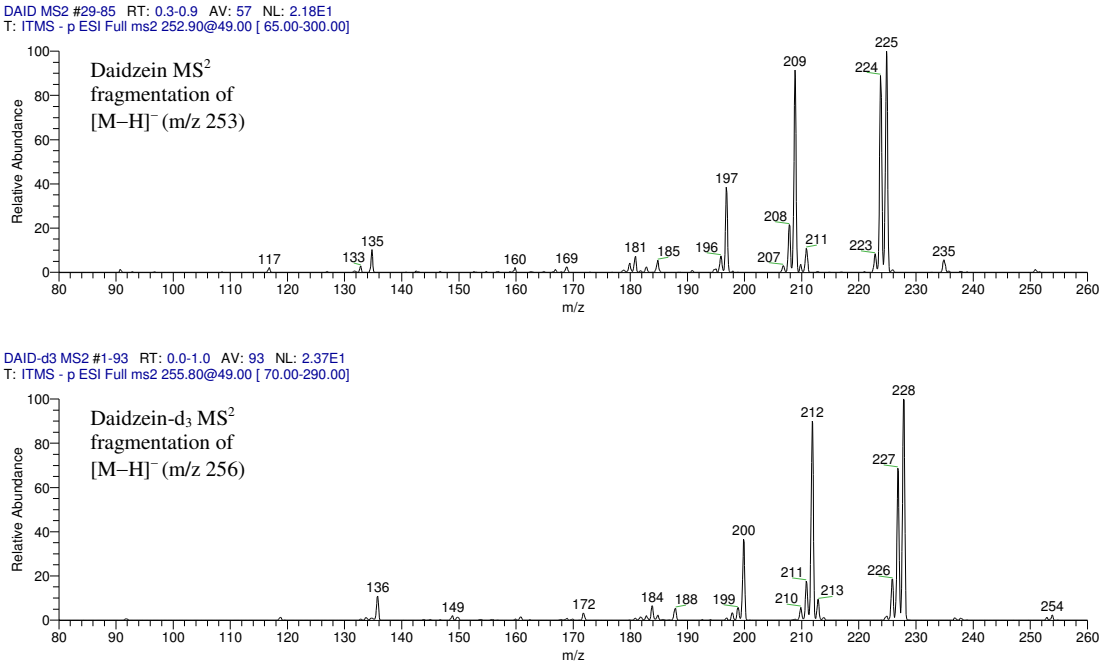
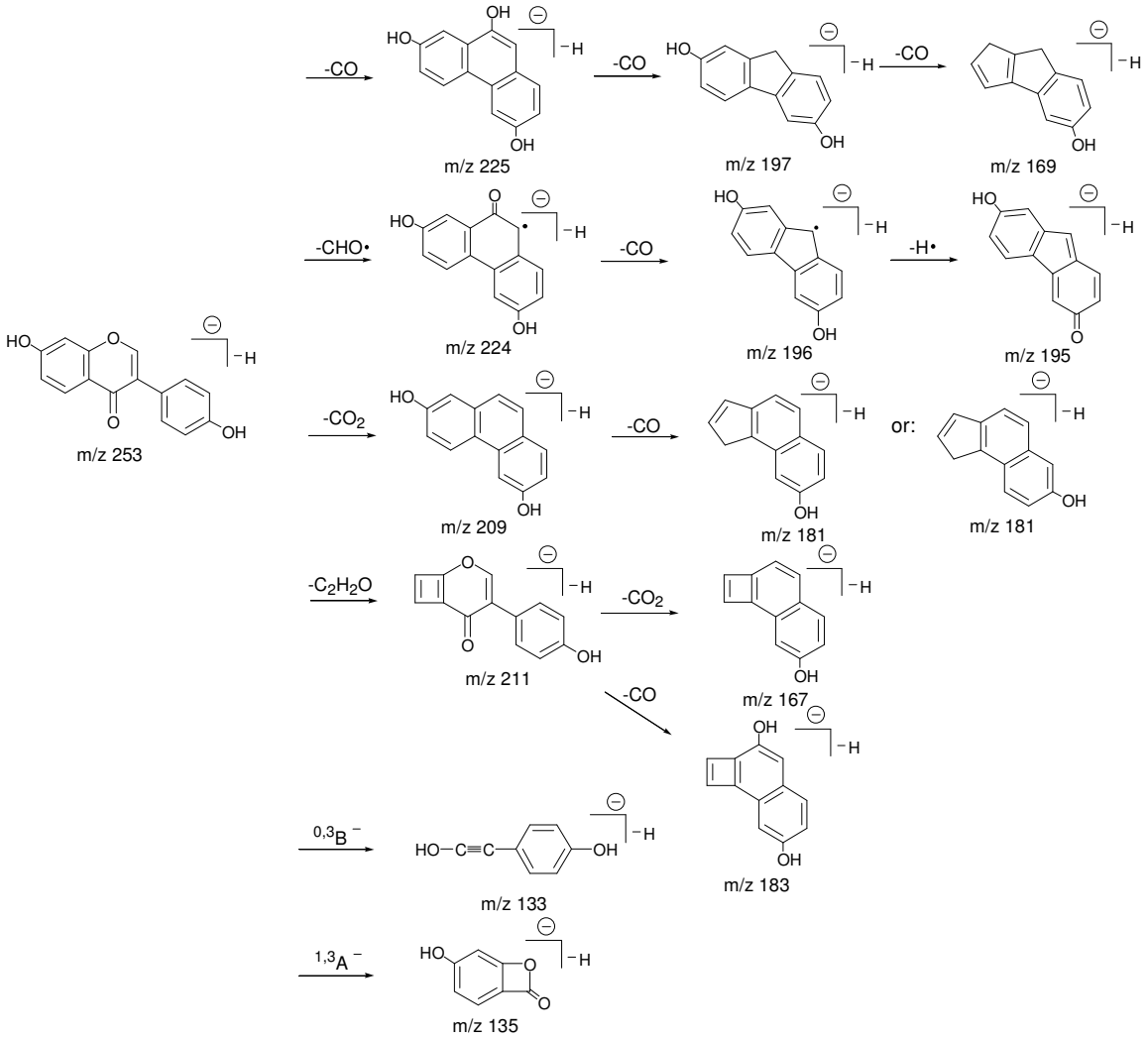



**Figure 5**. Fragmentation spectra of $[M–H]^-$ of compounds **3** and **4**.

**Table 2.** Product ions obtained from $[M–H]^-$ ions in ion trap $MS^2$ spectra for compounds **3** and **4** [*m/z* with relative abundances (%) in parenthesis].

| Ions | 3 | 4 |
|---|---|---|
| $[M–H–CO]^-$ | 225(100) | 228(100) |
| $[M–H–CHO]^{\cdot-}$ | 224(85) | 227(60) |
| $[M–H–C_2H_2O]^-$ | 211(10) | 213(10) |
| $[M–H–CO_2]^-$ | 209(90) | 212(90) |
| $[M–H–2CO]^-$ | 197(40) | 200(30) |
| $[M–H–CO_2–CO]^-$ | 181(5) | 184(2) |
| $^{0,3}B^-$ | 133(2) | 135(1) |
| $^{1,3}A^-$ | 135(5) | 136(5) |

*49% of the relative collision energy was used in the fragmentation.




**Scheme 5**. Proposed fragmentation scheme for daidzein in ESI- ion trap mass spectrometry.

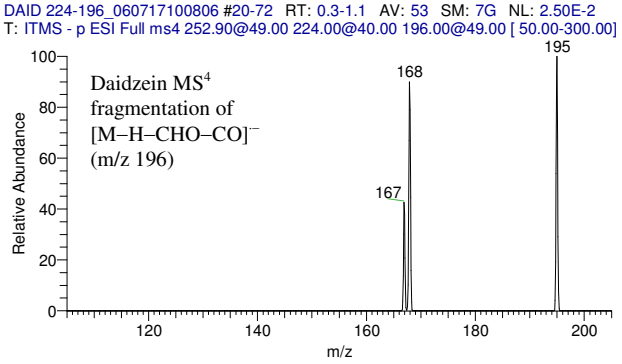



**Figure 6**. Fragmentation spectrum of m/z 196 of compound **3**.

**Fragmentation of biochanin-A, formononetin and glycitein by ion trap mass spectrometry**

Compounds **5** (biochanin A), **6** (formononetin), and **7** (glycitein) are methoxylated isoflavones. The $MS^2$ spectra of these three deprotonated methoxylated isoflavones all display only one abundant radical anion $[M–H–CH_3]^–$ peak by loss of a methyl group. This result is consistent with a previous study that showed that loss of $CH_3$· is a characteristic fragmentation

in methoxylated flavonoids.[13] In addition, loss of a hydrogen atom is easily found in these radical anions' following fragmentation spectra. Figure 7 shows fragmentation spectra of these three compounds, and Table 3–5 shows the proposed product ions observed in the $MS^n$ frgmentation spectra by ion trap mass spectrometry. The suggested fragmentation pathway and structures for $[M–H–CH_3]^{-}$ (m/z 268) of compound **5** (biochanin A) is shown in Scheme 6. The fragmentation behavior of this compound is different to that of the corresponding non-methoxylated one (genistein). The fragmentation spectrum of m/z 268 gives strong fragment peaks m/z 267 by losing a hydrogen atom; and also the $MS^4$ fragmentation spectra of m/z240 and m/z 224 both give strong peaks (m/z 239 and m/z 223) by loss of a hydrogen atom (see Figure 8). This can be explained by the possible formation of a more stable conjugated structure after loss of a hydrogen atom. The fragment ion peak (m/z 226) formed by loss of $C_2H_2O$ (–42u) is observed in the $MS^3$ fragmentation spectrum of $[M–H–CH_3]^{-}$ (see Figure 7), but no fragment peak loss of $C_3O_2$ (-68u) is found in all $MS^n$ fragmentation spectra of biochainin A, although it possesses a β-dihydroxy moiety in ring A.

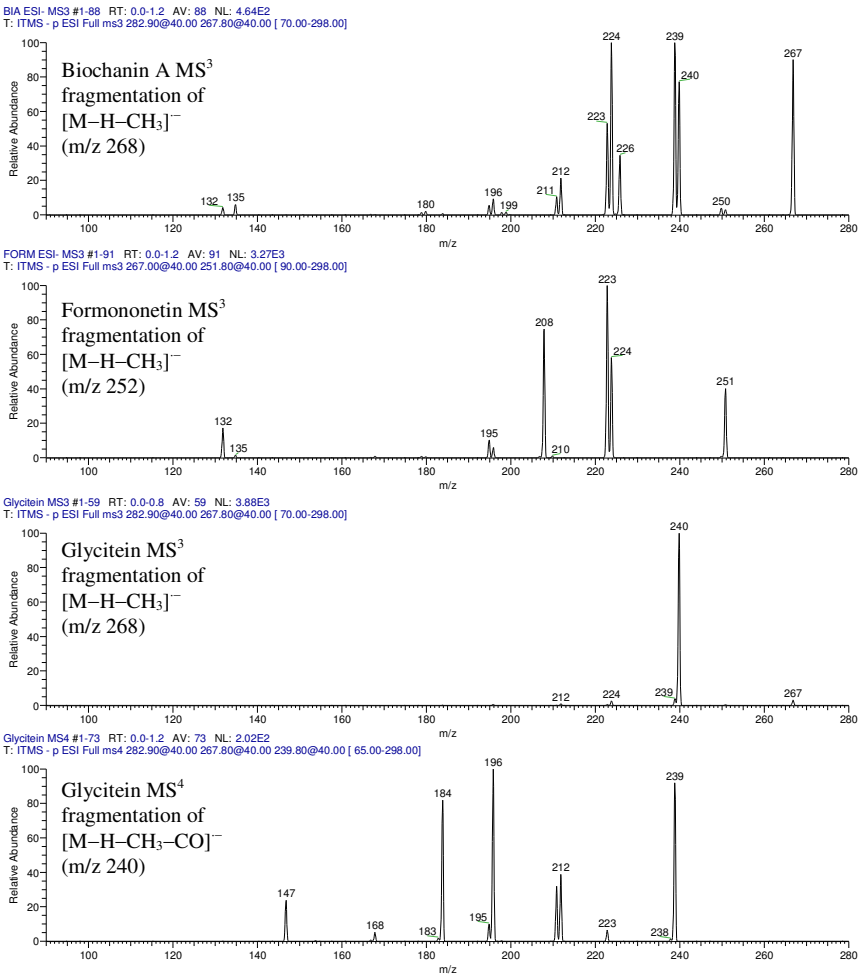



**Figure 7**. Fragmentation spectra of $[M–H–CH_3]^{-}$ of compounds **5**, **6**, **7**; and fragmentation spectrum of $[M–H–CH_3–CO]^{-}$ of compound **7**.

**Table 3.** Product ions obtained from $[M–H]^{-}$ ions in ion trap $MS^2$ spectra for compounds **5, 6, and 7** [*m/z* with relative abundances (%) in parenthesis].

| **Ions** | **5** | **6** | **7** |
|---|---|---|---|
| $[M–H–CH_3]^{-}$ | 268 (100) | 252 (100) | 268 (100) |

*40% of the relative collision energy was used in the fragmentation.

**Table 4.** Product ions obtained from $[M–H–CH_3]^{-}$ ions in ion trap $MS^3$ spectra for compounds **5, 6, and 7** [*m/z* with relative abundances (%) in parenthesis].

| Ions | 5 | 6 | 7 |
|---|---|---|---|
| $[M–H–CH_3–H]^{-}$ | 267 (90) | 251 (40) | 267 (2) |
| $[M–H–CH_3–CO]^{\cdot-}$ | 240 (80) | 224 (50) | 240 (100) |
| $[M–H–CH_3–CO_2]^{-}$ | 224 (100) | 208 (80) | — |
| $[M–H–CH_3–C_2H_2O]^{\cdot-}$ | 226 (30) | 210 (1) | — |
| $[M–H–CH_3–H–CO]^{-}$ | 239 (100) | 223 (100) | — |
| $[M–H–CH_3–H–CO_2]^{-}$ | 223 (50) | — | — |
| $[M–H–CH_3–2CO]^{\cdot-}$ | 212 (20) | 195 (10) | — |
| $[M–H–CH_3–CO_2–CO]^{\cdot-}$ | 196 (10) | — | — |
| [A-ring fragmment]$^{-}$ | 135 (5) | 135 (1) | — |
| [B-ring fragmment]$^{-}$ | 132 (2) | 132 (15) | — |

*40% of the relative collision energy was used in the fragmentation.

**Table 5.** Product ions obtained from $[M–H–CH_3–CO]^{-}$ ions in ion trap $MS^4$spectra for compounds **5, 6, and 7** [*m/z* with relative abundances (%) in parenthesis].

| Ions | 5 | 6 | 7 |
|---|---|---|---|
| $[M–H–CH_3–CO–H]^{-}$ | 239 (50) | 223 (10) | 239 (90) |
| $[M–H–CH_3–CO–CO]^{\cdot-}$ | 212 (40) | 196 (10) | 212 (40) |
| $[M–H–CH_3–CO–H–CO]^{-}$ | 211 (100) | 195 (100) | 211 (30) |
| $[M–H–CH_3–CO –CO_2]^{\cdot-}$ | 196 (20) | 180 (5) | 196 (100) |
| $[M–H–CH_3–CO–H–CO_2]^{-}$ | 195 (10) | — | |
| $[M–H–CH_3–3CO]^{\cdot-}$ | — | — | 184 (80) |
| $[M–H–CH_3–CO–CO_2–CO]^{\cdot-}$ | — | — | 168 (5) |
| $[M–H–CH_3–CO–B\text{-ring}]^{-}$ | — | — | 147 (25) |

*40% of the relative collision energy was used in the fragmentation.

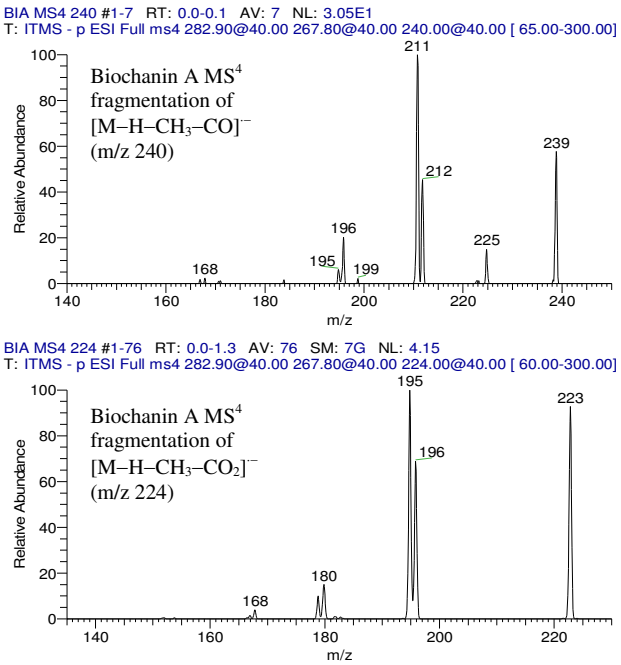
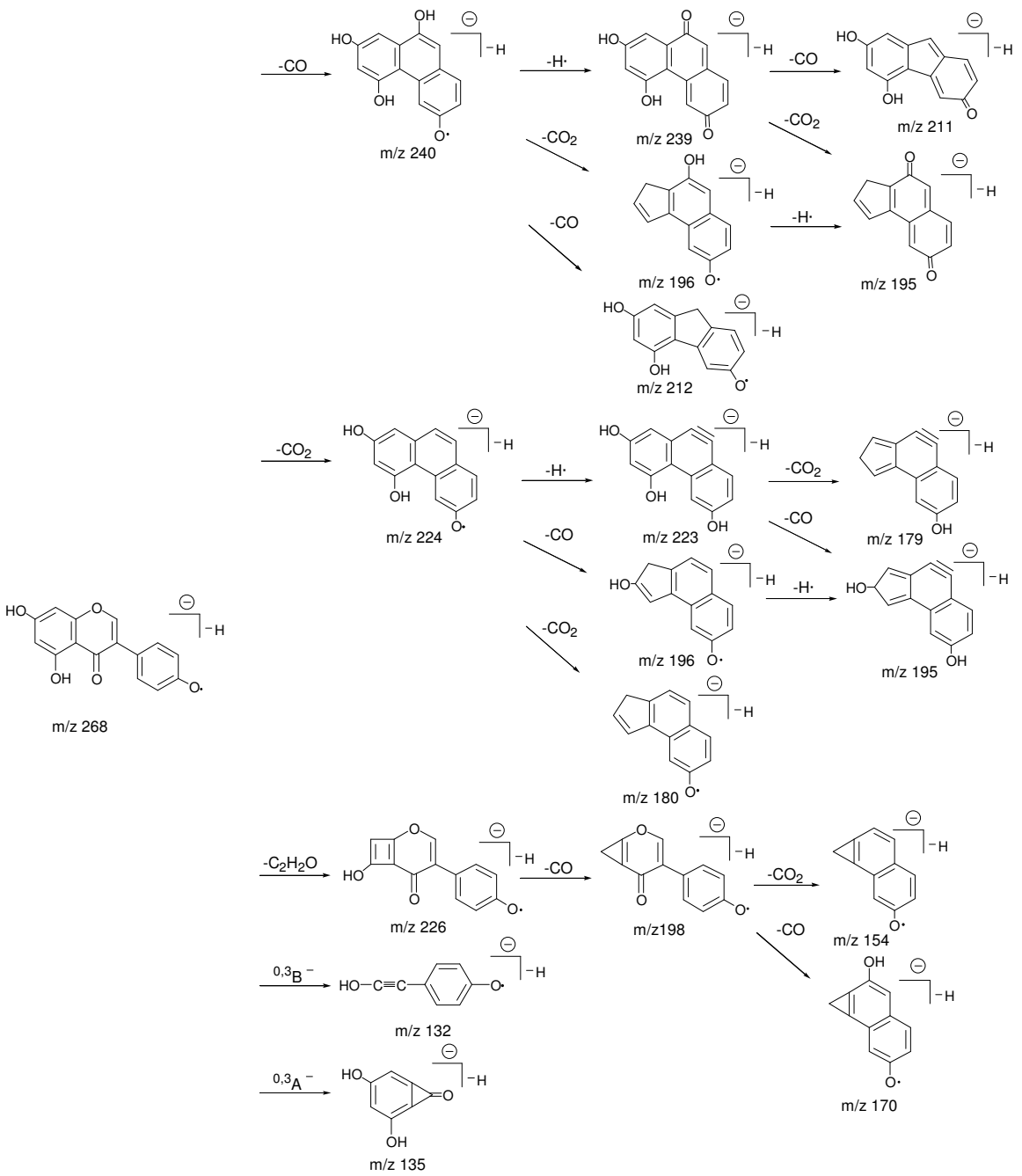
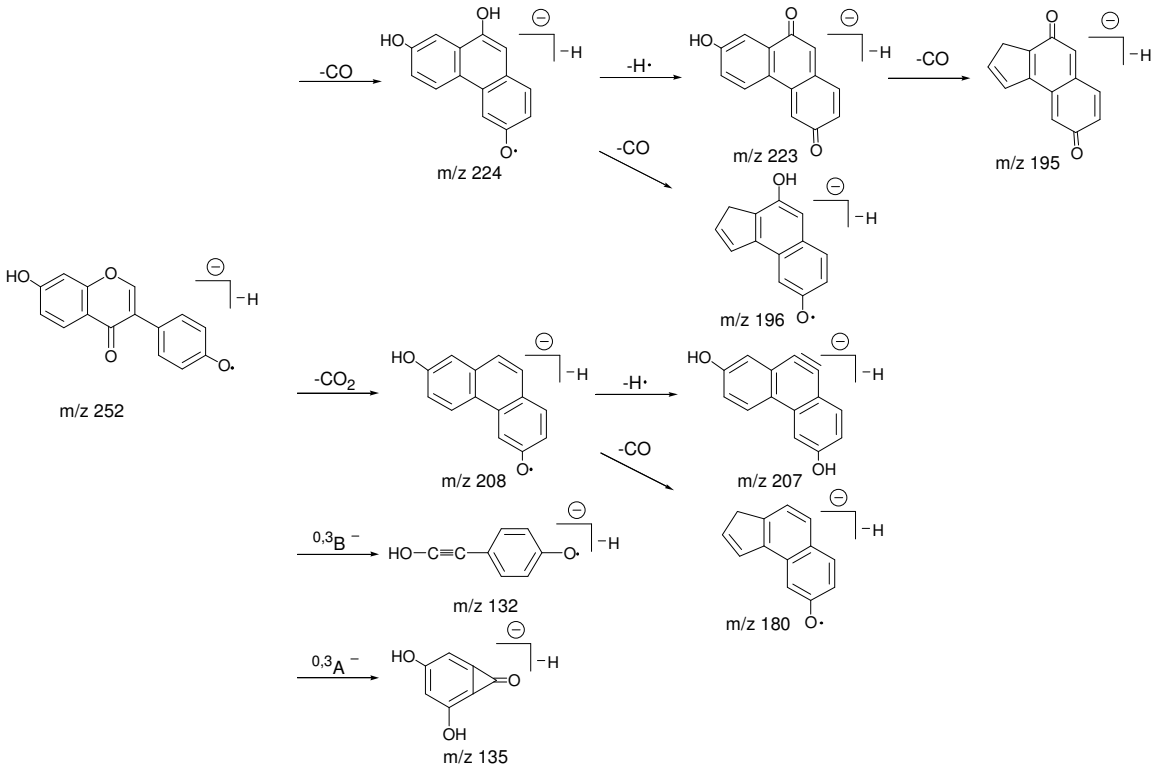



**Figure 8**. Fragmentation spectra of m/z 240 and m/z 224 of compound **5**.



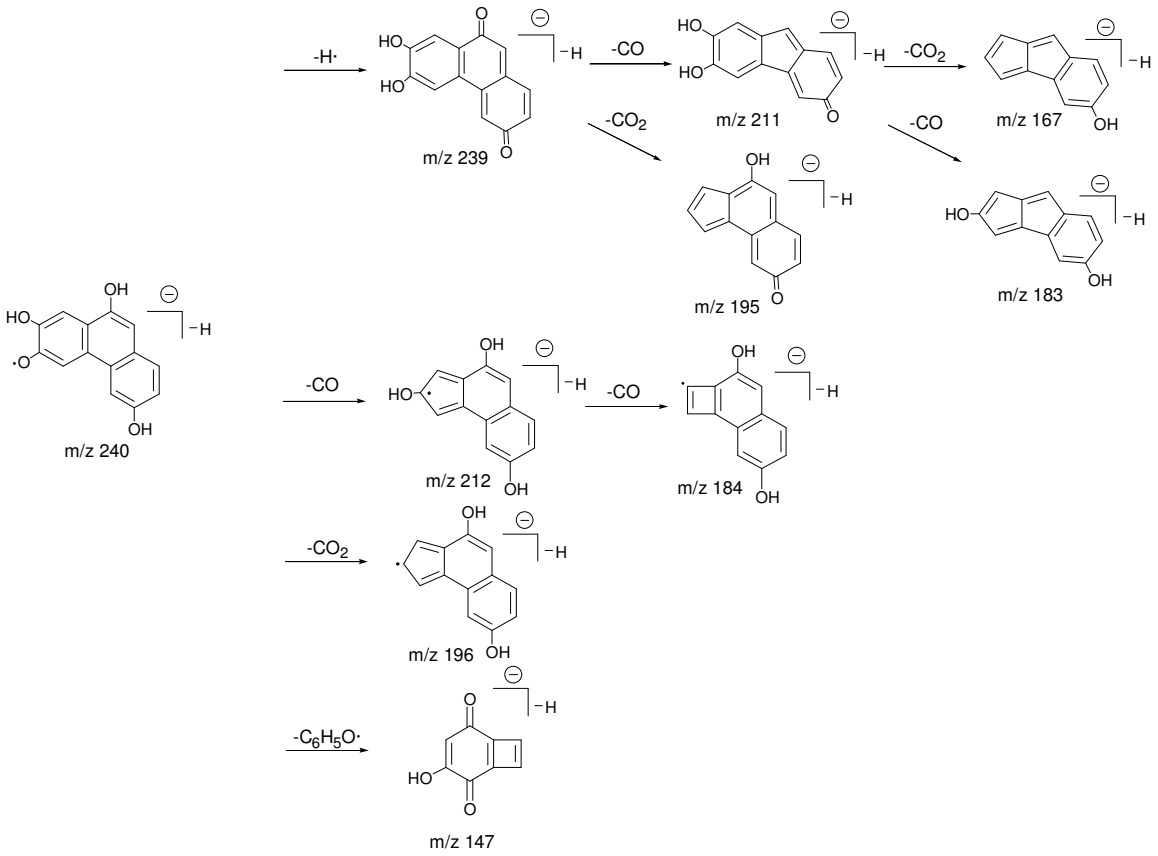

**Scheme 6**. Proposed fragmentation scheme for the m/z 268 radical anion of biochanin A by ESI- ion trap mass spectrometry.

Scheme 7 shows the fragmentation scheme for $[M–H–CH_3]^-$ ion (m/z 268) of compound **6** (formononetin). The fragmentation of formononetin is simpler than biochanin A as there is only one hydroxyl group on the ring A. Compared with the corresponding of non-methoxylated isoflavone, daidzein, stronger ion peaks with loss of hydrogen atom were observed with a very minor peak (1% of base peak) representing the loss of $C_2H_2O$ (–42u). As was the case with daidzein, no loss of $C_3O_2$ was found in all $MS^n$ spectra.




**Scheme 7**. Proposed fragmentation scheme for the m/z 252 radical anion of formononetin in ESI- ion trap mass spectrometry.

Compound **7** (glycitein) is an isomer of compound **5** (biochanin A), but their fragmentation behaviors are significantly different. The fragmentation scheme and fragment structures for $[M–H–CH_3–CO]^-$(m/z 240) are proposed in Scheme 8. Compared with biochanin A, the radical anon $[M–H–CH_3]^-$ undergoes facile loss of CO, its $MS^3$ spectrum giving a dominantly abundant base peak of $[M–H–CH_3–CO]^-$(m/z 240). Similar to the methoxylated isoflavones, loss of hydrogen atom is readily observed in the fragmentation spectra; the $MS^4$ fragmentation spectrum of m/z 240 gives a strong peak at m/z 239 (90% of the base peak, see Figure 7). Both m/z 240 and m/z 239 ion further lose CO to produce m/z 212 and m/z 211 ions respectively. It is interesting though that the $MS^5$ spectra of these two ions show much more divergence, the spectrum of m/z 211 ion gives a stronger fragment peak with loss of $CO_2$, but in the fragmentation spectrum of the m/z 212 ion the loss of $CO_2$ is hardly seen at all (see Figure 9); this result can be explained by the formation of these two ions, the loss of CO from the m/z 239 ion occurs at ring C and leaves two hydroxyl groups on ring A, whereas the loss of CO from the m/z 240 ion occurs at ring A and leaves no suitable structure for further loss of $CO_2$.




**Scheme 8**. Proposed fragmentation scheme for the m/z 240 radical anion of glycitein in ESI- ion trap mass spectrometry.

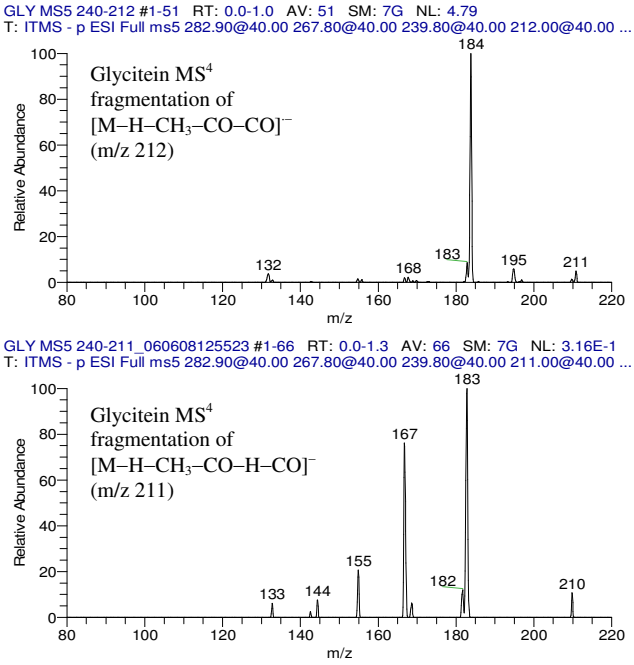
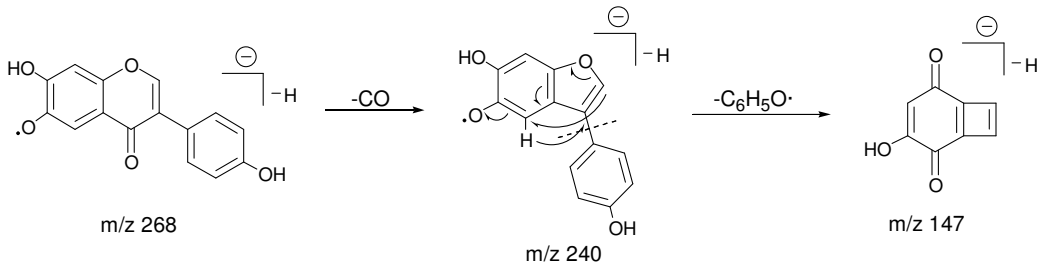



**Figure 9**. Fragmentation spectra of m/z 212 and m/z 211 of glycitein.

The most characteristic fragmentation ion of glycitein is $[M–H–CH_3–CO–B\text{-ring}]^-$ (m/z 147) formed by loss of a ring B radical (-93u). This could be attributed to the existence of a methoxyl group at $C_6$ of ring A and the subsequent formation of

an oxygen radical at this position remarkably influencing further fragmentation behavior. A proposed structure of m/z 147 and cleavage pathway is shown in Scheme 9.




**Scheme 9**. Proposed scheme for the formation of the m/z 147 ion.

**Fragmentation by triple quadrupole mass spectrometry**

The principal observed product ions obtained from $[M–H]^-$ ions of compounds **1** – **7** in the triple quadrupole mass spectrometry are shown in Table 6 – 8. Generally, the main fragmentation of the deprotonated isoflavones observed in the triple quadrupole mass spectrometry is the same as that seen in ion trap mass spectrometry, but there are also some differences. Unlike the fragmentation in the ion trap mass spectrometry, the tandem MS spectrum by the triple quadrupole mass spectrometry is the result of a one step process in which several fragment ions are found and where it is difficult to ascertain the relationship of the precursor and product ions. Compared with the fragmentation in ion trap mass spectrometry, a much higher abundance of fragment peak by recyclization cleavages are found on the MS tandem spectra in the triple quadrupole mass spectrometry. The most prominent product ion of compound **1** (genistein) is m/z 133 which is formed by a $^{0,3}B^-$ recyclization cleavage. Other recyclization cleavage products ($^{0,3}B^-–H·$, $^{0,3}A^-$) also are present in high abundance (see Table 6). In addition, the recyclization cleavage products for compound **3** (daidzein) also give strong peaks as well (see Table 7). Neutral loss of CO, $CO_2$, $C_3O_2$, and ketene are observed in the tandem MS fragmentation spectra (see Table 6 – 8).

**Table 6.** Principal product ions obtained from $[M–H]^-$ ions of compounds **1** and **2** by triple quadrupole mass spectrometry [*m/z* with relative abundances (%) in parenthesis].

| Ions | **1** | **2** |
|---|---|---|
| $^{0,3}B^-–H·$ | 132 (30) | 134 (35) |
| $^{0,3}B^-$ | 133 (100) | 135 (100) |
| $^{0,3}A^-$ | 135 (45) | 137 (35) |
| $[M–H–C_3O_2–C_2H_2O]^-$ | 159 (50) | 162 (45) |
| $[M–H–CO_2–H–CO_2]^-$ | 180 (40) | 184 (40) |
| $[M–H–C_3O_2]^-$ | 201 (20) | 205 (20) |
| $[M–H–CO_2–H]^-$ | 224 (30) | 228 (30) |
| $[M–H–CO–H]^-$ | 240 (25) | 244 (20) |

*Collision energy used in the fragmentation: 32eV.

In Table 6, the m/z 224 ion from compound **1** is unambiguously the result of loss $CO_2$ and a hydrogen atom. However, the formation of the m/z 240 ion may be by loss of either CO and a hydrogen atom or just a CHO·. We prefer the former because other product ions are found unambiguously by loss of a hydrogen atom, such as m/z 224 and m/z 180 from compound **1**; the corresponding ions are also observed in the deuterated compound **2**. The $[M–H–C_2O_3–C_2H_2O]^-$ ion (m/z 159) is observed from compound **1**, and the corresponding ion $[M–H–C_2O_3–C_2HDO]^-$ (m/z 162) is also observed from compound **2**. The information about loss of a deuterium atom in the ketene moiety from compound **2** supports the supposition that the loss of ketene occurs at ring A in isoflavones. In Table 7, the m/z 223 ion from compound **3** may be attributed to the loss of CHO· and a hydrogen atom (rather than the unlikely loss of CO and $H_2$); Similarly, the m/z 195 ion from compound **3** is formed by loss CO from m/z 223 rather than loss of two CHO· from $[M–H]^-$.

**Table 7.** Principal product ions obtained from $[M–H]^–$ ions from compounds **3** and **4** in triple quadrupole mass spectrometry [*m*/*z* with relative abundances (%) in parenthesis].

| Ions | **3** | **4** |
|---|---|---|
| $^{0,3}B^–$–H· | 132 (100) | 134 (100) |
| $^{0,3}B^–$ | 133 (65) | 135 (60) |
| $[M–H–CO_2–H–CO]^{·–}$ | 180 (50) | 183 (30) |
| $[M–H–CHO–H–CO]^–$ | 195 (60) | 198 (45) |
| $[M–H–CO_2–H]^{·–}$ | 208 (70) | 211 (60) |
| $[M–H–CHO–H]^–$ | 223 (90) | 226 (65) |

*Collision energy used in the fragmentation: 35eV.

The fragmentation spectra of the deprotonated methoxylated isoflavones (compounds **5**, **6**, and **7**) produce prominent peaks of $[M–H–CH_3]^{·–}$ radical anions by loss of a methyl group (see Table 8). This is consistent with Justesen[13] who observed this process for other deprotonated methoxylated flavonoids. In addition, the compound **7** (glycitein) displays a base peak at m/z 240 by further loss of CO from $[M–H–CH_3]^{·–}$ radical anion (m/z 268). This is consistent with the experiment by ion trap mass spectrometry, where the m/z 240 ion from compound 7 is almost the only abundant product ion from its precursor $[M–H–CH_3]^–$. Similar to the result in ion trap mass spectrometry, all three $[M–H–CH_3]^–$ give obvious $[M–H–CH_3–H]^–$ peaks by further loss of a hydrogen atom. This may be indicative that the loss of a hydrogen atom may be a common fragmentation feature for radical anions of isoflavones.

**Table 8.** Principal product ions obtained from $[M–H]^–$ ions of compounds **5, 6** and **7** by triple quadrupole mass spectrometry [*m*/*z* with relative abundances (%) in parenthesis].

| Ions | **5** | **6** | **7** |
|---|---|---|---|
| $^{0,3}B^–$–H· | 132 (10) | 132 (40) | — |
| $^{0,3}A^–$ | — | 135 (20) | — |
| $[M–H–CH_3–3CO]^{·–}$ | — | — | 184 (45) |
| $[M–H–CH_3–H–2CO]^–$ | — | 195 (40) | — |
| $[M–H–CH_3–CO–CO_2]^{·–}$ | — | — | 196 (40) |
| $[M–H–CH_3–H–2CO]^–$ | 211 (10) | — | 211 (20) |
| $[M–H–CH_3–H–CO_2]^–$ | 223 (10) | — | — |
| $[M–H–CH_3–H–CO]^–$ | 239 (20) | 223 (90) | 239 (20) |
| $[M–H–CH_3–CO]^{·–}$ | — | — | 240 (100) |
| $[M–H–CH_3–H]^–$ | 267 (30) | 251 (50) | 267 (10) |
| $[M–H–CH_3]^{·–}$ | 268 (100) | 252 (100) | 268 (60) |

*Collision energy used in the fragmentation: 30eV.

Overall, all the principal product ions observed in the triple quadrupole mass spectrometry are matched with the schemes and structures proposed from the ion trap mass spectrometry experiments, so it is not necessary to propose another scheme for the fragmentation in triple quadrupole mass spectrometry.

## CONCLUSIONS

In the present study, the fragmentation pathways of isoflavones (genistein, daidzein, biochanin A, formononitin, and glycitein) have been elucidated through $MS^n$ step fragmentation experiments in the ion trap mass spectrometer. With the aid of genistein-$d_4$ and daidzein-$d_3$, the location of a loss of ketene from the isoflavones is found to be at ring A. This is quite differenct to that reported previously for flavones and flavanones[9], where the loss of ketene is shown to occur at ring C. Tentative structures for the product ions are proposed based on their fragmentation behaviors and chemical intuition. Methoxylated isoflavones give only abundant peaks for the loss of a methyl group. Radical anions $[M–H–CH_3]^{·–}$ easily lose a hydrogen atom to form a more stable conjugated structure. Glycitein produces a characteristic fragment ion [M–H–

$CH_3$–CO–B-ring]$^-$ (m/z 147), which may result from the existence of a methoxy group at $C_6$. The ion trap spectrometry has a number of advantages over the triple quadrupole tandem mass spectrometry as the ion trap permits multiple step fragmentation experiments, which gives additional information to ascertain the relationship between precursor and product ions.

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